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Bovine articular cartilage decellularized matrix as a scaffold for use in cartilage tissue engineering

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Abstract

Extracellular matrix (ECM) - derived from mammalian tissues have been broadly used in tissue engineering both clinical and preclinical applications. While decellularization of tissues such as bladder, heart valve, knee meniscus and tendon has been performed, there has been a limitation to work with articular cartilage. This study aimed to develop a technique to decellularize bovine articular cartilage as a biological construct for cartilage substitution. For this purpose, alternative decellularization protocols including freeze/thaw cycles and treatment with various concentrations of sodium dodecyl sulfate (SDS) were used. Decellularization was analyzed by histological examination.Treatment with 2%SDS for 5 to 8h followed by4% SDS for3h hada significant effect on decellularization process. Treatment with 1% SDS had no effect on cell removal and use of 8% SDS for 5 to 8 hoursresulted in complete elimination of cells and significant decrease in cartilage matrix and collagen contents. This study provides a technique to produce acellular ECM derived from articular bovine cartilage whichmay serve as a xenogenic scaffold for cartilage tissue engineering.

Keywords: Bovine articular cartilage, decellularized matrix, sodium dodecyl sulfate, tissue engineering, tissue scaffold

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Introduction

Hyaline cartilage constitutes the important structure of synovial jointsand has a major role in the protection of articular surface of bone from abrasion and spreading the load over a larger surface of the joint. In addition, it provides the lubricated surface in order to facilitate the movement of joint and decrease friction (Buckwalter, 1983).On the other hand, hyaline cartilage can bear the repetitive physical stress as well. The complexity and the specialized structure of the hyaline cartilagehave brought up controversy to whether it canbe replaced and repaired once it is damaged (Furukawa *et al*., 1980). Treatments such as marrow stimulation techniques (microfractures) (Steadman *et al*., 1999), osteochondral autografts (Kish *et al*., 1999), harvested from cadaveric donors (Ghazavi *et al*., 1997) and autologous chondrocyte implantation (ACI) (Brittberg*et al*., 1994) would need cartilage scaffolds which can be cellularized in the recipients. Scaffolds constitute the principle component of tissue engineering applications which providea three dimensional substrate to support cell growth and differentiation. Biodegradable and bioresorbable biomaterials such as polylactic acid (PLA), polyglycolic acid (PGA) and collagen 1 as well as ECM derived scaffolds from decellularized tissues and organs have been used for cartilage tissue engineering applications. ECM- derived scaffolds could support cell growth due to its natural components and biocompatibility (Yang *et al*., 2008). Acellular scaffolds have been applied successfully in both preclinical and human applications (Gilbert *et al*., 2006). ECM- derived scaffold have been developed from tissues including heart valve (Schenke-Layland *et al*, 2003), skin (Chen *et al*., 2004), small intestine (Kropp *et al*., 1995), vascular tissues (Schmidt and Baier., 2000), tendons and ligaments (Woods and Gratzer., 2005). Physical and chemical treatments are the common methods for tissue decellularization procedures. Physical treatments include agitation, sonication, mechanical massage or pressure and freezing- thawto facilitate the removal of cellular components from ECM after disruption of cells membranes. Physical treatment must be coupled with chemical reagents in order to increase the effective decellularization protocols. Ionic detergents have been used to solubilize cell membranes. Biological tissues consist of cells and compact ECM. In order to remove cellsand cell debris during decellularization, ECM must be disrupted as well (Gilbert *et al*., 2006). The best decellularization protocols, however, are thosethat minimized ECM disruption and maintain mechanical and biological properties of the tissue. Little research has been done on ECM derived- cartilage scaffolds due to tissue compaction and difficulty of penetration of solutions used in the decellularization procedures (Yang *et al*., 2008). This study was aimed to characterize the effects of freeze/thaw cycle and different treatments of SDS on bovine cartilage in order to produce an acellular scaffold which could be further investigated for preclinical and clinical applications.

Materials and methods

Preparation of articular cartilage ECM

Articular cartilages of bovine femur bones were harvested from sacrificed animals immediately after slaughter. Then, the cartilage samples were cut in cylindrical shapes of 2 mm diameter. Tissue pieces were washed with phosphate-buffered saline (PBS) and were transferred to -4°C in order to begin physical stage of decellularization. Frozen pieces were thawed at room temperature (RT) and repeatedly washed with PBS. After that, specimens were immersed in liquid nitrogen for 2 min, thawed in distilled water and then were transferred to PBS at RT for 10 min. Last step was repeated 5 times. In chemical phase of decellularization, specimens were treated with different concentrations (1%, 2%, 4% and 8%) of sodium dodecyl sulfate (SDS, Merck) for 1, 3, 5 and 8 h at 37°C, while being gently agitated. In the next

step, cartilage segments were washed for 30 min in PBS at RT with gentle agitation. Then, in order to remove SDS, and to sterilize and eliminate remaining water from specimens, cartilage segments were placed in a sterile Buchner funnel (Ilmabop, Germany), washed with 75% ethanol, sterile distilled water and PBS, respectively.

Histological studies

Specimens were fixed at Bouin's solution, dehydrated through a graded series of ethanol, embedded in paraffin (Lab-O-Wax, Italy), and cross-sectioned at a thickness of 7 µm with microtome (Leits, Austria). Then, sections were deparaffinized, rehydrated and stained with one of the following techniques.

Hematoxylin & Eosin (H&E, Merck, Germany)

The sections were stained with the standard hematoxylin-eosin staining procedure.

The picrosirius red method

Picrosirius red dye was used to examine the effects of decellularization on the collagen-rich ECM. This is a specific histochemical dye for the detection of collagen in tissue sections. Briefly, this method consists of an initial staining in fresh Weigert's hematoxylin for 5 min, rinsing, and then staining of the sections in a 0.1% solution of Siriusred (Sirius Red F3B, Sigma) in saturated picric acid for 1 h (Junqueira *et al.*, 1979).When the stained sections are viewed with a microscope(Olympus, IX70, Japan), collagenous structures can be specifically and reliably identified (Junqueira *et al.*,1979).

Results

Articular cartilage (Fig.1) was decellularized with physicochemical methods including snap freezeing-thaw and treatment with sodium dodecyl sulfate (SDS). The effects of different concentrations of SDS for different periods on the decellularization process were monitored by hematoxylin-eosine staining (Fig. 2). Treatment with 1% and 2% SDS for 1h had no effect on the removal of cells, but4% and 8% SDS for 5 to 8h removed all the cells, while disrupted ECM. Lower concentrations of SDS for 5 to 8h followed by 4% and 8% SDS for 1 to 3h resulted in complete decellularization with maintaining ECM properties.

Collagen type II is a major component of bovine articular cartilage and provides important mechanical strength. The picrosirius-red staining was employed to evaluate the quality of collagen content in acellular articular cartilage. As shown in Fig. 3, collagen content was decreased by long treatments of ECM with higher concentrations of SDS, where ECM becomes disrupted. However, collagen content remained intact with lower concentrations of SDS in shorter periods of treatment. Histological techniques were employed to evaluate the decellularization of bovine articular cartilage. The results of H&E staining revealed that the articular cartilage did not have any cells in comparison with control samples (Fig. 1).

All cells were removed and only the empty sites of chondrocytes could be seen in tissue sections. Additionally, the results also indicated



Figure 1. Steromicroscopic view of bovine articular cartilage ×10.

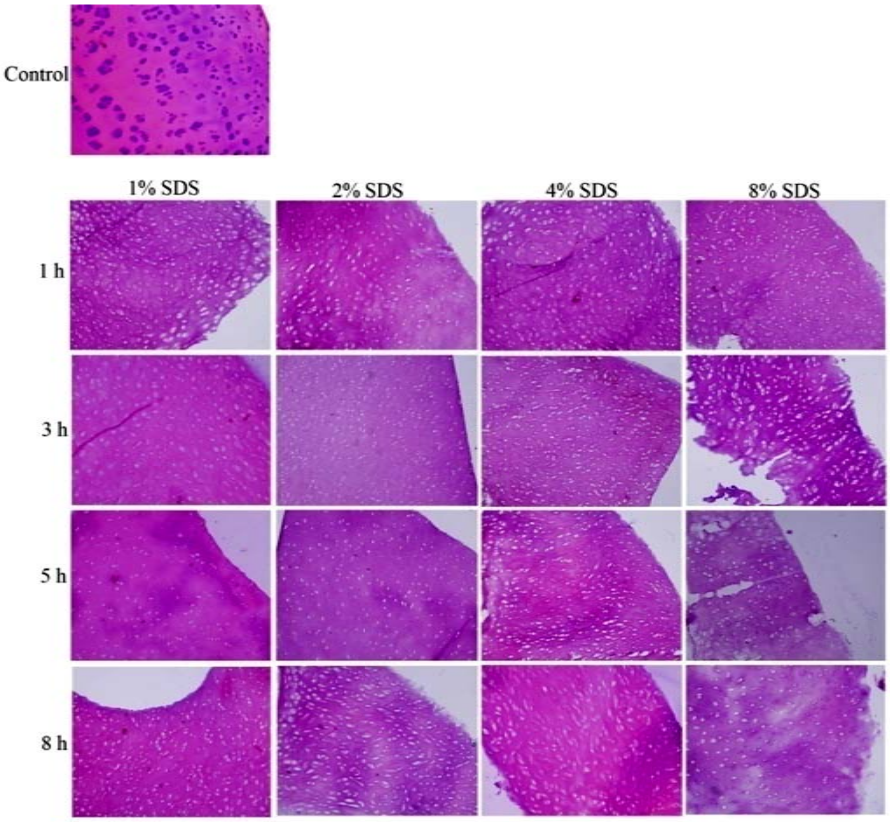


Figure 2. Control and decellularized cartilage samples after H&E staining. Chondrocytes are uniformly distributed through the cartilage. Insufficient decellularization is observed after 1h treatment with 1% and 2% SDS. Treatment with 8% SDS has caused the partial elimination of ECM. Treatment with 4% SDS for 1 to 5h followed by 2% SDS for a longer times could remove all cells, while ECM remains intact. ×100.

that ECM components have been preserved similar to control samples. Examination of collagen contents with picrosirius-red demonstrated that collagen fibers were also preserved in decellularized tissue.

Discussion

The objective of this study was to provide a protocol for decellularization of bovine articular cartilage with the aim of future tissue engineering applications. Tissue consist of cells and ECM, thus, an intact ECM would be the major goal in the tissue derived- ECM protocols.Thishas been the case for scaffolds derived from heart valves (Schenke-Layland et al. 2003), blood vessels (Schmidt and Baier., 2000), skin (Chen *et al*., 2004), Small intestine submucosal layer (Kropp *et al*., 1995), urinary bladder(Gilbert *et al*., 2005),tendons and ligaments (Woods and Gratzer., 2005). In a preliminary study to perform cartilage decellularization for clinical application, humantracheal cartilage has been treated with 4% SDS for 24h (Macchiarini *et al*., 2008). As the result of decellularization, porosity may appear which facilitates cell migration, mass transfer of nutrients and metabolic waste products (Yang *et al*., 2008). In this study, due to the very compact structure of cartilage and low penetrability of solutions, we used physicochemical methods to decellularize articular cartilage.

In the physical phase, samples were transferred to -4°C. This induces the formation of ice crystals, which can in turn disrupt chondrocytes and causes cell lysis. Cartilage scaffolds were then exposed to fast freezing in liquid nitrogen (-196 °C) causing more damage to cell membrane and removing proteins. As expected, the results indicated that these changes

of temperature caused minimal disruption in ECM. A dense meshwork of fibrous molecules was visible in the treated tissues. Kelley et al. attempted to use freezing-thaw in liquid nitrogen for decellularization of rabbit nasal septal cartilage, but cell fragments were still remained and caused immune reactions (Kelley *et al*., 2002). Thus, in this study SDS was used as a detergent for chemical phase of decellularization. Previous studies have revealed that SDS is the best detergent for removing cells and cell debris from tissues (Elder *et al*., 2010). With respect to amphipatic structure of SDS (a hydrophilic head and a hydrophobic tail), it can interact with cell membranes including plasma and nuclear membranes to lyses them. On the other hand, it can disrupt proteins by unfolding these molecules. As shown in Fig. 2 subjecting tissues to 1% SDS for 1 to 3h have insufficient effect on decellularization. However, it was shown that the higher levels of SDS concentration resulted in complete decellularization and produced a bovine articular cartilage matrix despite of deformation of the articular cartilage. There is a collagen network in cartilage matrix which has an important role in controlling the stresses and strains. These networks also provide the compressive properties of articular cartilage (Huber *et al*., 2000). Picrosirius- red staining was used as a special dye to determine collage quantities. This data showed that at the application of high levels of SDS, reduces collagen quantities and this may lead to decreasing mechanical properties of the ECM (Fig. 3). However, using 2% SDS for 3 to 5h followed by4% SDS for 1 to 3h resulted in complete decellularization, while matrix and collagen contents recompletely retained. Previous studies on porcine meniscus haverevealed that application of low level of SDS had no effect on collagen content.

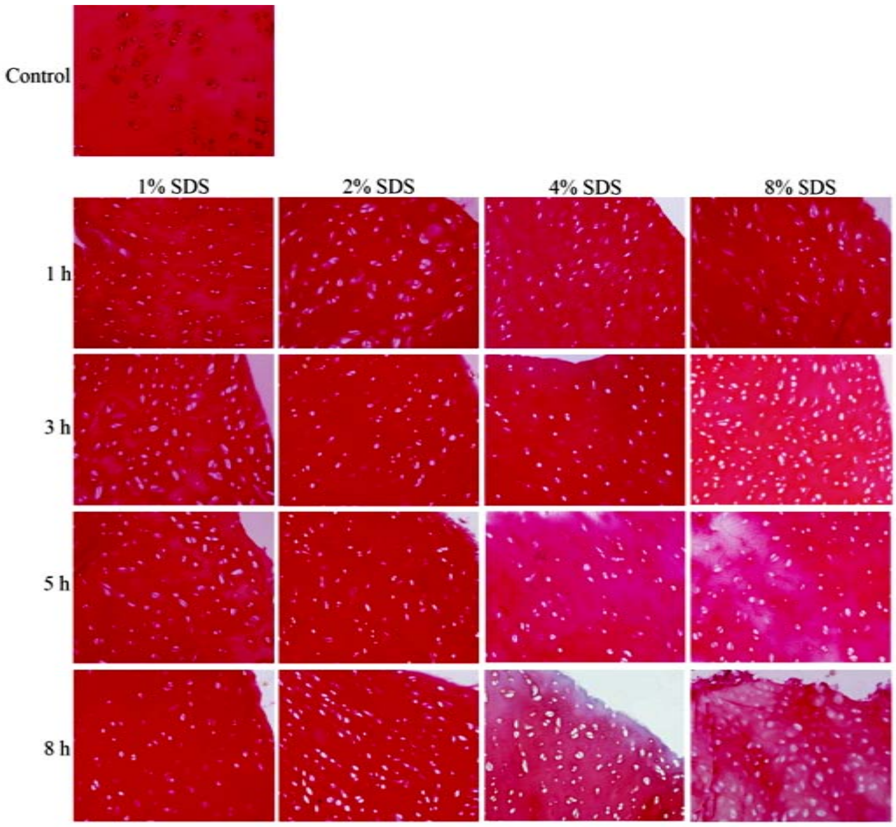


Figure 3. Control and decellularized cartilage samples after picrosirius-red staining. Control cartilage was shown at the top of photograph. Use of 4% and 8% SDS for 8 to 5h could reduce collagen content that results in a decrease in mechanical properties of the decellularized articular cartilage. ×100.

In conclusion, acellular biological scaffolds may have important applications in clinical implantation and regenerative operations. This acellular scaffold may be recellularized by endogenous cells or autologus chondrocytes. Further studies are required to investigate the regenerative (recellularization) capacity of the scaffold in animal models.

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چکیده

مواد مشتق شده از ماتریکس خارج سلولی پستانداران به طور گسترده ای در مدل های حیوانی در حیطه مهندسی بافت مورد استفاده قرار گرفته اند. با وجود اینکه فرایند سلول زدایی در بافت هایی از قبیل مثانه، دریچه قلبی، مینیسک زانو و تاندون صورت گرفته است گزارشات اندکی دال بر سلول زدایی در بافت غضروف مفصلی وجود دارد. در این مطالعه هدف معرفی روشی برای سلول زدایی از غضروف مفصلی گاو بوده تا بتوان به عنوان داربستی زیستی برای جایگزین های غضروف مورد استفاده قرار داد. به منظور دستیابی به این هدف، روش های مختلف سلول زدایی از قبیل سیکل های فریز- انجماد و به دنبال آن تیمار با غلظت های مختلف SDS مورد استفاده قرار گرفت. حذف سلول ها توسط تکنیک های بافت شناسی از قبیل رنگ آمیزی هماتوکسیلین- ائوزین و پیکروسیروس رد مورد ارزیابی قرار گرفت. تیمار با SDS ۲٪ به مدت ۵ تا ۸ ساعت و همچنین SDS ۴٪ در مدت زمان ۳ ساعت بیشترین تاثیر را در حذف سلول ها با حداقل اثر بر روی محتوای کلاژنی داشته و این در حالی است که در تیمار با SDS ۱٪ سلول ها حذف نشدند و از طرفیSDS ۸٪ در مدت زمان ۵ تا ۸ ساعت منجربه حذف کامل سلول ها از بافت مورد نظر شد ولی به دنبال آن کاهشی قابل توجه در محتویات کلاژن مشاهده گردید. این مطالعه روشی را برای حذف سلول ها از غضروف مفصلی گاو به منظور ایجاد ماتریکسی سلول زدایی فراهم کرده که می تواند به عنوان داربستی زنوژنیک در مطالعات مهندسی بافت مورد استفاده قرار گیرد.

واژگان کلیدی: غضروف مفصلی گاو، ماتریکس سلول زدایی شده، سدیم دودسیل سولفات، مهندسی بافت